

## Comparison of eight *Pisolithus tinctorius* isolates for growth rate, enzyme activity, and phytohormone production

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Received April 29, 1986

Accepted September 3, 1986

Ho, I. 1987. Comparison of eight *Pisolithus tinctorius* isolates for growth rate, enzyme activity, and phytohormone production. Can. J. For. Res. 17: 31–35.

Eight isolates of *Pisolithus tinctorius* (Pers.) Coker and Couch (three each from Georgia and northern California and one each from Oregon and Washington) were compared *in vitro* for growth rate, for alkaline and acid phosphatase and nitrate reductase activities, for acid phosphatase isozyme patterns, and for cytokinin, indoleacetic acid, and gibberellin production. Significant differences appeared between isolates for each parameter examined. All isolates showed relatively low phosphatase and nitrate reductase activities. Isolate S-359 from northern California grew the slowest in culture and produced significantly more indoleacetic acid than all other isolates and more cytokinin than six of the other seven isolates; this isolate was also the only one of the eight that did not share at least one acid phosphatase allele with the others in the isozyme analysis.

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Chez huit isolats du *Pisolithus tinctorius* (Pers.) Coker et Couch (trois chacun pour la Géorgie et le nord de la Californie et un chacun pour Washington et l'Orégon), l'auteur a comparé le taux de croissance, les activités des phosphatases alcalines et acides ainsi que de la nitrate réductase, et enfin les productions d'hormones, cytokinines, acide indole acétique et gibbérellines. Pour chacun des paramètres examinés il y a des différences significatives. Tous les isolats montrent des activités phosphatases et nitrate réductases relativement faibles. L'isolat S-359 du nord de la Californie pousse plus lentement en culture et produit significativement plus d'acide indole acétique que tous les autres isolats et plus de cytokinine que six des sept autres isolats; ce dernier est aussi le seul des huit qui, d'après l'analyse des isoenzymes ne partage pas au moins un allèle pour la phosphatase.

[Traduit par la revue]

### Introduction

*Pisolithus tinctorius* is distributed around the world and has been recorded from at least 36 states in the United States (Grand 1976). It can form ectomycorrhizae with a broad range of hosts (Marx 1977) in habitats such as mine spoils, sand dunes, sawdust piles, and eroded or rocky and shallow soils. These sites are characterized by environmental extremes, such as high soil temperature in summer, extreme acidity, drought, low fertility, and high concentrations of heavy metals (Schramm 1966; Marx *et al.* 1982).

Isolates of *P. tinctorius* differ markedly in culture characteristics and in effectiveness as inocula in both bare-root and container nurseries (Molina 1979; Marx 1981). To obtain a better understanding of such differences, I compared eight isolates from the west coast and from Georgia in the U.S.A. for growth rate, enzyme activity, acid phosphatase isozyme patterns, and phytohormone production.

### Materials and methods

Eight isolates from sporocarps were maintained as stock cultures at the Forestry Sciences Laboratory, Corvallis (Table 1); three (S-172, S-210, and S-360) had been previously used by Molina (1979) in mycorrhizal inoculation experiments.

Analytical methods for monoester and diester acid and alkaline phosphatases, nitrate reductase, acid phosphatase isozymes, cytokinins, indoleacetic acid (IAA), and gibberellins were as given below.

#### *Fungus culture and experimental design*

Isolates were grown in 250-mL Erlenmeyer flasks with 100 mL of liquid medium for enzyme activity determination, and in 1-L Erlenmeyer flasks with 500 mL of medium for analysis of free growth regulators. The liquid medium was modified Melin–Norkrans (MN) solution: 0.05 g of CaCl<sub>2</sub>, 0.025 g of NaCl, 0.5 g of KH<sub>2</sub>PO<sub>4</sub>, 0.25 g of (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.15 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.02 g of sequestrene (Geigy), 100 µg of thiamine HCl, and 5 g of dextrose per litre of distilled H<sub>2</sub>O.

The medium was adjusted to pH 5.5 and autoclaved at 115°C at 15 lb (1 lb = 0.454 kg). Five replicate flasks for each isolate for each set of analyses including separate set for measuring growth rate were inoculated with plugs removed with a 3 mm diameter brass loop from the edges of 1-month-old colonies on MN agar plates. All treatments were arranged in a completely randomized design in room temperature with subdued light during the day and no added light during the night; the data were subjected to analysis of variance.

#### *Phosphatase activity*

After a month of growth at room temperature, the colony was filtered from each flask and washed aseptically with sterile deionized water followed by a rinse with sterile universal buffer (Skujin *et al.* 1962). A stock solution of universal buffer was made of 12.1 g of tris(hydroxymethyl)aminomethane, 11.6 g of maleic acid, 14.0 g of citric acid, 6.28 g of boric acid, and 488 mL of 1 N NaOH, with distilled water added to bring the total volume to 1 L. Stock solution (20 mL) was adjusted to pH 5.5 after dilution for acid phosphatase and pH 10 for alkaline phosphatase determination (Eivazi and Tabatabai 1976), and distilled water was added to bring the volume to 100 mL. The buffer solution was then sterilized by Millipore filtration (0.22 µm pore size). After the aseptic rinse with sterile universal buffer, each colony was placed in a sterile 20-mL screw-cap tube with 4 mL of buffer solution plus 1 mL filter-sterilized, 0.1 M disodium-*p*-nitrophenyl phosphate and incubated in a 30°C water bath for 2 h in the dark. After the tubes were incubated, 5 mL of 0.5 M NaOH were added to stop the reaction. The colony was removed, rinsed, and oven-dried at 60°C for 24 h. Acid and alkaline phosphatase activity of separate sets of samples was determined from the amount of *p*-nitrophenol released in the filtrate from the phosphatase substrate as measured in Perkin–Elmer 552A Vis/UV spectrophotometer<sup>1</sup> at 410 nm. The substrate for diesterphosphatase was bis-*p*-nitrophenyl phosphatase (Eivazi and Tabatabai 1976); otherwise, its analysis was the same as for monoester phosphatase.

<sup>1</sup>Use of trade names does not constitute approval by the United States Department of Agriculture Forest Service.

TABLE 1. Sources of isolates of *Pisolithus tinctorius* examined for enzyme activity and phytohormone production

State	County	Isolate	Associated host genera	Elevation (m)	Year of collection
Georgia		S-181	<i>Pinus taeda</i>		1974
		S-210	<i>P. taeda</i>		1976
		S-471	<i>P. taeda</i>		1978
California	Siskiyou	S-359	<i>P. lambertiana</i>	330	1976
		S-360	<i>Lithocarpus densiflorus</i> , <i>Arbutus menziesii</i>	600	1976
	Trinity	S-370	<i>Pseudotsuga menziesii</i>	400	1976
Oregon	Benton	A-172	<i>Quercus borealis</i>	200	1976
Washington	Skamania	S-431	<i>P. menziesii</i>	350	1978

NOTE: Experiments were conducted in 1980–1982.

#### Gel-electrophoretic analysis

Each colony was washed in distilled sterile 0.1 M Tris–acetate buffer to remove liquid culture medium and then suspended in same buffer at pH 6.8, containing 5% of PVP 40, 5% of DOW X1, 1% Triton X100, and 1% 2-mercaptoethanol, and stored at 0°C.

Starch gel electrophoresis was performed according to methods of Scandalios (1969) and Conkle *et al.* (1982). The colony was ground in buffer solution, and the suspension was absorbed into a 5 × 10 mm paper wick. The gel was prepared from 128 g of hydrolyzed starch with 1066 mL of gel buffer solution (960 mL of 0.2 M Tris–citrate buffer at pH 8.3 and 106 mL of 0.2 M lithium–borate buffer at pH 8.3). The test was conducted at a constant current of 100 mA for 4 h. The developing solution for detecting acid phosphatase isozymes consisted of 200 mL of 0.2 M acetate buffer at pH 4, 100 mg of  $\alpha$ -naphthyl acid phosphate, 100 mg of fast garnet GBC, and 10 drops of 10% MgCl<sub>2</sub> solution. The isozymes were recorded after 3 and 24 h.

#### Nitrate reductase activity

Each culture was filtered by using No. 1 filter paper and washed aseptically with sterile deionized water, followed by a rinse with sterile phosphate buffer at pH 7.0. After the rinse, each colony was aseptically transferred to a sterile 20-mL screw-cap tube containing 3 mL of filter-sterilized phosphate buffer at pH 7.0, 1 mL of 0.1 M neutral succinic acid, and 1 mL of 0.1 M KNO<sub>3</sub>. The tubes were incubated in a vacuum incubator at 30°C for 4 h. After incubation, tubes were removed and the contents mixed with 2.5 mL of 0.02% *N*-(1-naphthyl)-ethylenediamine dihydrochloride and 2.5 mL of 1% sulfanilamide in 10% HCl (Ho and Trappe 1980). Amount of nitrate present in each mixture was determined on a Vis/UV spectrophotometer at 540 nm.

#### Indolacetic acid

The isolates were grown in 500 mL of modified Melin–Norkran's liquid medium. Culture filtrates were acidified to pH 2.0 and partitioned against reagent-grade methylene chloride in a separatory funnel. The methylene chloride portions were dried in a rotary evaporator. The residues were dissolved in 10 mL of ethanol for ultraviolet (UV) spectrophotometric determination at 280 nm (Galston and Davies 1970).

Thin-layer chromatography was carried out by the methods of Obreiter and Stowe (1964), with 20 × 20 cm glass plates coated with silicic acid – carboxymethyl cellulose (28.5:1.5, by weight). The solvent system was *n*-butanol – chloroform (3:2, v/v) saturated with 0.5 M formic acid. Ehrlich's detection reagent was prepared by adding 2% (w/v) *p*-dimethylaminobenzaldehyde to a mixture of absolute ethanol and 10 N HCl (1:1, v/v).

#### Cytokinins

Each colony was filtered from 500 mL of modified Melin–Norkran's liquid medium. Filtrates were adjusted to pH 2.0 and extracted with reagent-grade methylene chloride three times. The water phase was then adjusted to pH 8.6 with 1 N NaOH and extracted with two volumes

TABLE 2. Growth of eight isolates of *Pisolithus tinctorius* for 1 month at room temperature

Isolate	Ovendried weight
Georgia	
S-181	0.52a
S-210	0.40ab
S-471	0.27bcd
California	
S-359	0.05d
S-360	0.34abc
S-370	0.17cd
Oregon	
A-172	0.22bcd
Washington	
S-431	0.06d

NOTE: Isolates not sharing a common letter differ significantly ( $P < 0.05$ ) by Tukey's test for differences among treatment means.

of ethyl acetate three times. The ethyl acetate combined fraction was evaporated to dryness under vacuum at 37°C and the residue dissolved in 80% ethanol. The ethanol extract was reduced to water phase under vacuum at 37°C and then extracted with two volumes of ethyl acetate three times. The ethyl acetate fraction was discarded, and the water fraction adjusted to pH 5.5 and extracted with three volumes of water-saturated *n*-butanol three times. The water fraction was discarded. This procedure purportedly removes all other plant-growth substances, such as auxins, gibberellins, and abscisic acid (Shindy and Smith 1975). The *n*-butanol fractions were vacuum dried and the residues taken up in 10 mL of deionized water for UV spectrophotometric measurement at 254 nm. Thin-layer chromatography was carried out on 20 × 20 cm glass plates, precoated with silica gel to a thickness of 1 mm. Each extract was applied as a streak. The solvent system for separation was *n*-butanol – 1 N NH<sub>4</sub>OH – water (Carlson and Larson 1977). Zeatin and zeatin riboside (Sigma) used as cytokinin standards were spotted at both edges of the plates. The plates were air dried at the end of the test.

#### Gibberellins

Free gibberellins were extracted by the procedure of Gaskin and MacMillan (1978). Culture filtrates were partitioned at pH 8 with ethyl acetate for neutral and basic ethyl acetate fractions (nonpolar gibberellic acids such as GA<sub>9</sub>, GA glucosyl esters), again partitioned at pH 2.5 with ethyl acetate for the acidic ethyl acetate fraction (free GAs), and finally partitioned at pH 2.5 with *n*-butanol for the acidic *n*-butanol

TABLE 3. Acid and alkaline phosphatase activity of eight isolates of *Pisolithus tinctorius* expressed as millimoles per gram of fungal dry weight

Isolate	Acid monoester phosphatase	Alkaline monoester phosphatase	Ratio	Acid diester phosphatase	Alkaline diester phosphatase	Ratio
Georgia						
S-181	18.4 <sub>a</sub>	1.2 <sub>a</sub>	15.4 <sub>a</sub>	4.1 <sub>a</sub>	1.7 <sub>b</sub>	2.4 <sub>a</sub>
S-210	3.9 <sub>bc</sub>	2.3 <sub>a</sub>	1.7 <sub>bc</sub>	0.5 <sub>c</sub>	1.3 <sub>b</sub>	0.4 <sub>c</sub>
S-471	2.9 <sub>bc</sub>	1.2 <sub>a</sub>	2.4 <sub>bc</sub>	1.3 <sub>bc</sub>	1.2 <sub>b</sub>	1.1 <sub>bc</sub>
California						
S-359	4.3 <sub>bc</sub>	1.9 <sub>a</sub>	2.2 <sub>bc</sub>	0.9 <sub>c</sub>	1.2 <sub>b</sub>	0.8 <sub>bc</sub>
S-360	7.3 <sub>b</sub>	3.2 <sub>a</sub>	2.3 <sub>bc</sub>	0.8 <sub>c</sub>	0.9 <sub>b</sub>	0.9 <sub>bc</sub>
S-370	3.8 <sub>bc</sub>	1.2 <sub>a</sub>	3.1 <sub>b</sub>	3.5 <sub>ab</sub>	3.7 <sub>a</sub>	0.9 <sub>bc</sub>
Oregon						
A-172	3.6 <sub>bc</sub>	3.8 <sub>a</sub>	1.0 <sub>c</sub>	1.7 <sub>bc</sub>	1.0 <sub>b</sub>	1.7 <sub>b</sub>
Washington						
S-431	2.0 <sub>c</sub>	1.4 <sub>a</sub>	1.4 <sub>bc</sub>	0.8 <sub>c</sub>	0.9 <sub>b</sub>	0.9 <sub>bc</sub>

NOTE: Isolates not sharing a common letter differ significantly ( $P < 0.05$ ) by Tukey's test for differences among treatment means.

fraction (polar GAs such as GA<sub>32</sub>, GA glucosyl esters). The extracts were concentrated under reduced pressure at low temperature. The remaining traces of water were removed by azeotropic distillation in toluene. Finally, the extracts were esterified (Reeve and Crozier 1978). Extracts were dried over phosphorus pentoxide *in vacuo* for 6 h; dioxane and dimethylformamide dibenzylacetol were added through the septum at the rate of 100 mm<sup>3</sup>/m of samples and heated in a 70°C water bath up to 4 h for complete esterification. At the moment the reaction was completed, GA benzyl esters separated from the excess of dimethylformamide dibenzylacetol. The extracts and standards were measured at 254 nm on a UV spectrophotometer.

## Results and discussion

### Growth rate

No geographic or host patterns in growth rate were identified (Table 2). Georgia and West Coast isolates both included representatives of the significantly fastest and slowest growing isolates. The same was true when isolates associated with Pinaceae and Fagaceae were compared. The isolates used by Molina (1979) grew at the same rate in relation to each other in both his and my studies. No relationship between rate of growth in agar medium and capacity of isolates or reisolates to form ectomycorrhizae was reported (Marx 1981).

### Phosphatases

Isolates of *P. tinctorius* showed much lower acid and alkaline phosphatase activities than *Rhizopogon* species, *Laccaria laccata* (I. Ho and J. M. Trappe, unpublished data), and other ectomycorrhizal fungi (Ho and Zak 1979). Beckjord *et al.* (1984) found that both roots and leaves of nonmycorrhizal seedlings contained significantly more phosphorus than those of *P. tinctorius* inoculated seedlings. The weak phosphatase activity of this fungus (Table 3) may account for ineffective phosphorus uptake in such circumstances.

*Pisolithus tinctorius* has proved especially effective as a symbiont of pines in harsh sites with low organic matter (Ruehle and Marx 1979). In forest soils of the Pacific Northwest which typically abound in organic matter, *P. tinctorius* has generally not promoted survival and growth of seedlings in the many plantations in which it has been tested (M. A. Castellano and J. M. Trappe, unpublished data). I hypothesize that its relatively poor capacity to produce phosphatases limits its competitive ability where organophosphorus is a major phosphorus source. Release and utilization of organophosphorus by trees consumes

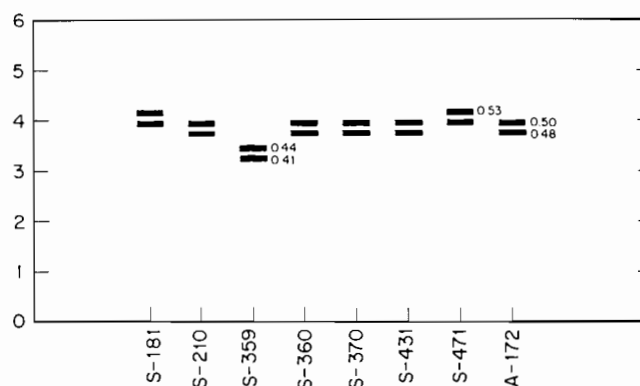


FIG. 1. Zymogram of acid phosphatase isozymes of eight isolates of *Pisolithus tinctorius*.

both energy and nitrogen. *Pisolithus tinctorius* may benefit its host more by mycelial growth than by enzyme production. Spreading of mycelia through a large volume of soil and coming in contact with available, mineralized nutrients could be advantageous in low organic soils. In high organic soils, fungi with higher phosphatase and decompositional capacity might have a competitive advantage in obtaining nutrients.

### Isozymes of acid phosphatase

The isozymes of acid phosphatase separated readily in starch-gel electrophoresis. All isolates except S-359 from California shared at least one allele (Fig. 1), which indicates greater similarity in acid phosphatase loci than I have found in other ectomycorrhizal fungi (unpublished data).

### Nitrate reductase

Nitrate reductase activity of the eight isolates of *P. tinctorius* (Table 4) was generally lower than that reported for other ectomycorrhizal fungi (Ho and Trappe 1980). The fastest growing isolate showed the highest nitrate reductase activity. The lower nitrate reduction capacity may reflect the adaptability and preference for soil chemical conditions in which this fungus thrives.

### Phytohormones

The eight isolates of *P. tinctorius* produced various amounts of IAA, cytokinins, and gibberellins (Table 5). The effects of these phytohormones on host plants will be discussed in future

TABLE 4. Nitrate reductase activity expressed as micromoles per gram of fungal dry weight

Isolate	Nitrate reductase activity
Georgia	
S-181	28.1 <sub>a</sub>
S-210	2.7 <sub>b</sub>
S-471	2.1 <sub>b</sub>
California	
S-359	1.3 <sub>b</sub>
S-360	1.6 <sub>b</sub>
S-370	0.7 <sub>b</sub>
Oregon	
A-172	1.0 <sub>b</sub>
Washington	
S-431	0.8 <sub>b</sub>

NOTE: Isolates not sharing a common letter differ significantly ( $P < 0.05$ ) by Tukey's test for differences among treatment means.

TABLE 5. Phytohormones of eight isolates of *P. tinctorius* expressed as micromoles per gram of fungal dry weight

Isolate	Cytokinin	IAA	Cy/IAA	Gibberellins
Georgia				
S-181	22.1 <sub>c</sub>	68.2 <sub>b</sub>	0.3 <sub>b</sub>	5.2 <sub>c</sub>
S-210	28.4 <sub>c</sub>	56.0 <sub>b</sub>	0.5 <sub>b</sub>	8.5 <sub>bc</sub>
S-471	60.9 <sub>abc</sub>	124.8 <sub>b</sub>	0.5 <sub>b</sub>	15.1 <sub>b</sub>
California				
S-359	259.1 <sub>ab</sub>	1045.4 <sub>a</sub>	0.2 <sub>b</sub>	19.3 <sub>b</sub>
S-360	51.3 <sub>bc</sub>	64.3 <sub>b</sub>	0.8 <sub>b</sub>	9.7 <sub>bc</sub>
S-370	66.3 <sub>abc</sub>	335.4 <sub>b</sub>	0.2 <sub>b</sub>	18.8 <sub>b</sub>
Oregon				
A-192	63.7 <sub>abc</sub>	246.2 <sub>b</sub>	0.3 <sub>b</sub>	13.9 <sub>bc</sub>
Washington				
S-431	271.4 <sub>a</sub>	139.9 <sub>b</sub>	1.9 <sub>a</sub>	44.3 <sub>a</sub>

NOTE: Isolates not sharing a common letter differ significantly ( $P < 0.05$ ) by Tukey's test for differences among treatment means.

papers on *Rhizopogon* species, *Tricholoma magnivelare* (Peck) Redhead, and other ectomycorrhizal fungi. The phytohormones strongly influence the morphology of mycorrhizae (Slankis 1973, 1974; Graham and Linderman 1980, 1981; Rupp and Mudge 1984). Variability in ectomycorrhizal development among isolates of *P. tinctorius* has been reported by Molina (1979) and Marx (1981). These variations may be related to the hormones liberated by the mycelium of *P. tinctorius* (Navratil and Rochon 1981). S-359 from northern California produced significantly higher amounts of IAA than other isolates; it also differed from the others in acid phosphatase isozyme patterns. S-431 from Washington showed high amounts of cytokinin and the significantly highest cytokinin : IAA ratio; it also produced the most gibberellins (Table 4).

### Conclusions

Enzyme and phytohormone activity differed considerably among different isolates of *P. tinctorius*, confirming the need to evaluate many isolates of mycorrhizal fungi in selecting specific isolates for nursery inoculation. The significance of these *in vitro* variations to successful inoculation and desired host

response now needs to be experimentally determined in fungus–host–soil systems.

### Acknowledgements

I thank Dr. Donald Copes for valuable suggestions and advice, Dr. James M. Trappe for his critical review of the manuscript, Dr. Randy Molina for his assistance in mycorrhizal culture, and Ms. Margaret De La Rosa for help in laboratory work.

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